Investigation into the Intestinal Microflora of Children with Autism Spectrum Disorder

Kristy M Klug¹; Maureen E Geraghty, PhD, RD, LD¹; Lingling Wang, PhD²; Zhongtang Yu, PhD²; Jody L Wall, RD, LD¹; Alison Lane, PhD, OTR/L³

¹ Medical Dietetics Division, School of Allied Medical Professions, The Ohio State University; ² Department of Animal Sciences, The Ohio State University; ³ Occupational Therapy Division, School of Allied Medical Professions, The Ohio State University

Address correspondence to Maureen Geraghty, Medical Dietetics Division, The Ohio State University, 306L Atwell Hall, 453 West 10th Ave, Columbus, OH 43210
Office: (614) 247-4595
Fax: (614) 202-0210
Maureen.geraghty@osumc.edu
Abstract

Objectives: Autism Spectrum Disorders (ASD) continue to be diagnosed at a rapidly progressing rate now estimated to be 1 in 91 children in the United States. Autism is a pervasive developmental disorder characterized by a range of atypical behaviors that include difficulties with social reciprocity, impaired language development, resistance to change, and repetitive behaviors. About 40-70% of children diagnosed with ASD suffer from gastrointestinal distress, impairing proper nutrient consumption, which has been associated with abnormalities in their intestinal microflora. The primary objective of the study was to quantify and compare both beneficial (Bifidobacteria, Lactobacilli) and pathogenic (Clostridia) bacteria in children aged 3-9 with ASDs as compared to neurotypical controls. Methods: Participants were selected based on the eligibility criteria prohibiting antibiotics or probiotics. Molecular characterization of the stool samples included real-time polymerase chain reactions (PCR) assays for total Bifidobacterium, Lactobacillus, and Clostridium (C. clusters I, XIV, and C. bolteae).

Results: Unpaired t-tests of the molecular characterization of ASD stool samples revealed significantly lower amounts of Lactobacilli (p = 0.0059) and higher amounts of C. bolteae (p = 0.0079) than that of their typical peers. No significant differences were found in the levels of Bifidobacteria (p=0.2066), C. cluster I (p = 0.9391), and C. cluster XIV (p = 0.187) between the two groups. Conclusions: Results suggest that children with autism may have significantly lower amounts of beneficial (Lactobacilli) bacteria and higher amounts of pathogenic bacteria (C. bolteae). Further study is warranted to determine whether interventions such as prebiotic or probiotic supplementation would be advantageous in improving intestinal microflora.

Key words: autism, gastrointestinal microflora in autism, developmental disabilities
CHAPTER 1
Introduction

1. Problem Statement
2. Review of Literature
3. Objectives

CHAPTER 2
Materials and Methods

1. Methodology
2. Population and Sample
3. Design
4. Data and Instrumentation

CHAPTER 3
Results
Discussion

REFERENCES
CHAPTER 1

INTRODUCTION

1. Problem Statement

Many parents of children with autism spectrum disorder (ASD) frequently report gastrointestinal distress in this population. Children with autism may have insufficient amounts of beneficial intestinal microflora, which suggests excessive amounts of harmful microflora, compared to neurotypical children. Understanding the composition of this population’s intestinal microflora may lead to improvements in the treatment of autism, as well as reveal information for further research in the field of autism. With estimates indicating 1 in 91 children are impacted by this disorder (1), this subject is of national importance.

2. Review of Literature

Autism: An Overview

Autism spectrum disorder is a condition characterized by a range of atypical behaviors that include difficulties with social interactions, impaired language development, resistance to change, and repetitive behaviors. Autism often creates an unstable and stressful environment for the affected child and his or her family members (2). Although autism is characterized by a highly variable prognosis for children, “most children with an early diagnosis of autism are not completely independent as adults and the disorder generally has lifelong effects on the child’s ability to be social, to care for themselves, and to participate in the community (2).” With no clear etiology, we are not equipped with effective means of prevention or treatments (2). Without a cure, we rely on the most recent research to lend insight into this increasingly
prevalent disorder so as to develop promising outcomes for the future of these affected children (2).

It has been reported that many autistic children experience a variety of gastrointestinal disorders (3, 4). Studies have revealed an abnormal composition of intestinal microflora in children with autism (2-7). Of these studies examined, half of them documented the apparent association of the parallel onset of behavioral autistic symptoms with gastrointestinal complications (4-6, 8) as a result of the presence of compromised intestinal microflora (5, 6). These findings have provided a basis for further research into the connection among gastrointestinal complications, altered intestinal microflora, malabsorption, and the onset of behavioral characteristics of autism spectrum disorder in this population (9).

**Gastrointestinal Dysfunction and Behavioral Symptoms Manifested in Autism**

Many research articles have revealed gastrointestinal abnormalities in children diagnosed with autism spectrum disorder. The symptoms reported include constipation, diarrhea, flatulence/bloating, gastroesophageal reflux, abdominal pain and gastrointestinal discomfort (5, 8, 10). Parents have noted the onset of behavior symptoms of autism and the above noted gastrointestinal complications to manifest themselves concurrently (4-6, 8), “however, until recently, gastrointestinal symptoms of these children received little attention (8, p. 560).” Almost twenty years after that publication, although many advances in this research field have been accomplished, we are still struggling to find the link between the gastrointestinal symptoms and ASD.

Jyonouchi et al (10) investigated the relationship between an elimination diet (such as casein-free/gluten-free) and improvement in the gastrointestinal symptoms as well as apparent improvement of some behavioral symptoms of children with ASD. They studied the association
of dietary proteins and innate immune response of 72 children with autism compared to children with dietary protein intolerances and unrelated healthy children. Their results indicated an abnormal (inflammatory) innate immune response to dietary proteins play a role in gastrointestinal inflammation and subsequent gastrointestinal symptoms of ASD children. Furthermore, speculations that chronic gastrointestinal complications exacerbate some behavioral symptoms of autistic children, secondary to gastrointestinal discomfort, were revealed (10).

Parracho et al (5) investigated the association of gastrointestinal problems and the indigenous gut flora of children with ASD. Researchers disclosed that while “food intolerance is suspected to play a role in ASDs, the underlying cause of gastrointestinal symptoms remains unclear (5).” The study found that gastrointestinal distress was associated with increased levels of clostridia, toxin-producing bacteria, in children with ASD. Additionally, the study acknowledged the apparent worsening of behavioral symptoms during periods of gastrointestinal complications. Researchers attributed this connection to the possible systemic effects of increased toxins, including neurotoxins, in the bloodstream from high levels of clostridia in the autistic gut (5).

In a recently published medical hypothesis, Finegold reports that his review of literature suggests that “intestinal bacteria are a factor in autism in that autistic children showed improvement in behavior, communication, and social skills and in the gastrointestinal manifestations of their disease after oral vancomycin, an antibiotic that is essentially not absorbed from the gut (6),” was administered. In fact, the researchers from this study, Sandler et al (11), found that eight of ten autistic children studied exhibited marked improvement during therapy. However, “although improvement was clear by several measures (11),” the study
reported all but one autistic child regressed back towards baseline following the termination of the antibiotic. Despite the fact that this study showed short-term improvement in behavioral autistic symptoms as a result of antibiotics not recommended for the treatment of autism, it has suggested a link between the gut flora and the brain activity of a subset of autistic children. The investigators encouraged “further research of a possible gut-brain connection be vigorously pursued (11).”

**Intestinal Microflora Associated with Autism and its Significance**

After the link between autism and gastrointestinal complications was acknowledged as a key aspect of this idiopathic disorder, research began addressing the etiology of the gastrointestinal symptoms and their possible implications in autism spectrum disorder. It was established early on that high frequencies of autistic children express altered (increased) gut permeability (4). Researchers then developed interest in the composition of the intrinsic microflora of autistic children, providing the basis for current autism research (3, 5-7).

Clostridia, a neurotoxin-producing, endospore-forming harmful bacterial species, have been documented to compromise the intrinsic microflora of autistic children compared to neurotypical children (3, 5-7, 11). Furthermore, this organism is speculated to be responsible for, or at least contribute to the exacerbation of behavioral characteristics of autism (5, 6).

Finegold et al published a study that revealed significantly higher counts and species of clostridia in the microflora of autistic children compared to those of neurotypical control children using traditionally cultured (plate-based method) specimens (7). This group then produced a similar study, wherein they used real-time polymerase chain reaction (real-time-PCR) method due to the known limitations of significantly underestimating bacteria present in fecal samples using the traditional method (3). Data collected in the latter study found that cell count
differences between the autistic and control children were statistically significant, revealing a 46-fold elevation in *Clostridia bolteae*, a 9.0-fold elevation in *Clostridia clusters I*, and a 3.5-fold elevation in *Clostridia clusters XI* in autistic children compared to the control children (3). Song et al (3) discuss the efficacy of real-time-PCR to reveal that “the findings in this study by real-time PCR are in agreement with the previous observations and provide reliable quantitative information (3).” The researchers then report that “investigation of the intestinal microflora is crucial for obtaining an understanding of the role of the gut microflora in health and also understanding the role of the microflora in disease (3).”

Following these studies, Parracho et al recognized “numerical differences in gut flora of ASD patients and healthy subjects remain poorly studied (5),” and implemented their own investigation. Their results indicated a significant relationship between autism and gastrointestinal symptoms. Furthermore, those gastrointestinal complications were associated with increased levels of clostridia, suggesting an additional association with the development of certain autistic characteristics (5). These findings support the integral role the gut microflora plays in autism (5). Researchers suggest “modulation of the gut microflora by reducing the numbers of certain clostridia in ASD patients, while stimulating more beneficial gut bacteria, may help alleviate some of the related symptoms (5).”

There is a clear implication throughout the literature of the importance of additional research in the field of autism to determine the role of gastrointestinal symptomatology and related autistic behaviors (9). The prevalence of gastrointestinal symptomatology in children with ASD has a consensus of about 30-40%, with some estimates extending to 80% (9). There are also speculated contributions of suboptimal gut microflora to these gastrointestinal
complications. Geraghty et al (9) also describe the theory of altered intestinal permeability ("leaky gut") which has been suggested to contribute to the characteristics of autism (9).

Based on the information gathered from this preliminary study, future research may include the use of prebiotics and probiotics in children with autism. These dietary supplements (prebiotics) promote the growth of the beneficial microorganisms, specifically, Bifidobacterium and Lactobacillus. The beneficial microorganisms are considered to have a protective effect on the gut integrity by preventing and inhibiting the growth of pathogenic bacteria (9).

**Conclusion**

Autism is a devastating disorder, not only to the children affected, but the parents and family members as well. These children suffer from social and language skill deficits, as well as restrictive and repetitive patterns of activity. With prevalence as high as 1 in 91, and no cure in sight, the need for research in this field is imperative.

The gastrointestinal complications seen in autism have been associated with possibly affecting the development of certain autistic characteristics and behaviors. These gastrointestinal symptoms have been linked to the compromised intestinal microflora of autistic children. With the sophisticated techniques used in research these days, such as real-time polymerase chain reactions as well as the improved DNA extraction method published by Drs. Yu and Morrison (12), there is great potential for advancements in the understanding of and the treatment for children with autism spectrum disorder.

Preliminary studies have obviated the necessity for further research into the connection between the autistic gut and the onset of autistic symptoms. Research in this field may lead to discoveries related to the etiology of, symptoms related to, and potential treatments for autism spectrum disorders. A recently developed research plan exhibits great potential for the future of
autistic children by embracing the already published knowledge, and implementing a plan to potentially correct the suboptimal gastrointestinal composition (13).

Autism spectrum disorder is a multifactorial disorder not well understood, even today. However, as research advances, we may reveal knowledge that gives these children and families potential for a brighter future.

3. Objectives

The purpose of this study is intended to address two specific aims:

- **Specific Aim 1:** To determine if lower quantities of beneficial microflora (*Bifidobacteria* and *Lactobacilli*) exists among children with autism spectrum disorder compared to neurotypical children, compromising the gut integrity of these individuals.

- **Specific Aim 2:** To determine if higher quantities of pathogenic microflora (*Clostridia*) exists among children with autism spectrum disorder compared to neurotypical children, compromising the gut integrity of these individuals.

**Hypothesis:** Children with autism spectrum disorder will possess significantly fewer counts of *Bifidobacteria* and *Lactobacilli*, and greater counts of the pathogenic family of bacteria, *Clostridia*, than those of neurotypical children.
CHAPTER 2

METHODS AND MATERIALS

This research is in conjunction with: Lane, A, Case-Smith, J, Geraghty, M. Factors predictive of problem eating behaviors in children with autism (IRB #2008H0315). This study implemented consent-informed procedures as approved by the IRB in the OSU Office of Responsible Research Practices. IRB approval dated February 16, 2009 has been obtained.

1. Population and Sample

Participants – Participants were selected based on a convenience sample for this pilot study. Thirteen children aged from 3 to 9 years diagnosed with autism spectrum disorder (includes: autism, Asperger’s syndrome, Pervasive Developmental Disorder (PDD) and Pervasive Developmental Disorder – Not Otherwise Specified (PDD-NOS)) were recruited to this pilot study. An additional ten typically developing (neurotypical) children without immediate family members with any related ASD were recruited to compare intestinal microflora with children with autistic children. Eligibility criteria for all children prohibited the consumption of yogurt for the past (one) month, antibiotics for the past three months, and probiotic supplements for the past six months. Neurotypical children were healthy and free of gastrointestinal diagnoses or symptoms.

2. Design

Recruitment Procedure – Eligible children with autism were recruited from relevant clinics at the Ohio State University Medical Center’s Nisonger Center and through established autism networks and support groups in Ohio. Clinic coordinators at the Nisonger Center assisted in identifying eligible children. Where the potential autistic child study participant is a past client of the clinic, families were mailed a letter of invitation to participate including information
about the study. New clients presenting to the clinics were provided with study information by clinic coordinators once their eligibility had been determined. IRB approved Informed Consent procedures and documents were employed. Eligible neurotypical potential participants were recruited via OSU Child Care, broadcast emails to OSU faculty and staff, and personal contacts in Ohio.

**Study procedure** – All neurotypical children and autistic participants were asked to supply a stool sample during an initial home visit. An additional visit was scheduled to pick up and transfer the sample to a minus-eighty (-80 C) freezer as soon as possible so as to preserve the sample. Participants were provided with a Stool kit, comprised of a fitted plastic toilet seat (in which to “catch” the specimen), a labeled 15 mL conical Falcon® tube in which to place the stool sample, plastic gloves, and a sterilized wooden mini-spatula. They were also provided with detailed stool collection instructions.

This student’s (KK) role and responsibilities included: (1) recruitment of participants, (2) dropping off Stool kits and collecting stool samples from selected subjects at a time negotiated with the participants’ parent, (3) several hours of observing and aiding a lab assistant with DNA extraction and real-time polymerase chain reaction (real-time-PCR) method, and (4) collecting and analyzing data from the real-time-PCR assays.

**3. Methodology**

**DNA extraction and real-time PCR assays.** Microbial community DNA was extracted from 0.5 g of each stool sample using the repeated bead-beading +column (RBB+C) method, an improved method of DNA extraction published by Drs. Yu and Morrison, in whose lab we conducted our molecular work (12). The absolute abundance (AA) of *Bifidobacteria, Lactobacilli, Clostridia* Group I, *Clostridia* Group III and *C. bolteae* 16S *rrs* genes was quantified against each
respective standard using the group-specific real-time PCR assays. The relative abundance (RA) is expressed as the number of each group (or species) 16S rrs copies per million copies (cpmc) of total bacterial 16S RNA genes. The relative abundance was calculated by dividing the absolute abundance of each group (or species) by the total bacterial absolute abundance in each sample and then by multiplying by 1 million (RA = AA / total bacterial AA X 1 million). The primers and probes used in this study are listed in Table 1. Sample-derived standards were prepared for each real-time PCR assay using a composite DNA sample pooled from all the samples to be analyzed (14, 15). The real-time PCR assays were carried out following the condition described previously (14, 15). Total bacterial 16S rRNA genes were also quantified using a universal primer set and a universal TaqMan probe (14). All the real-time PCR assays were done in triplicate for each standard and microbiome DNA sample.

### Table 1. Sequences of oligonucleotide primer and probes

<table>
<thead>
<tr>
<th>Target bacteria and probe or primer</th>
<th>Oligonucleotide sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
<th>°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe-I</td>
<td>6-FAM-5’- CGT ATT ACC GCG GCT GCT GGC AC-3’- TAMRA</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gBifid-F</td>
<td>CTC CTG GAA ACG GGT GG</td>
<td>549-563</td>
<td>55°C</td>
</tr>
<tr>
<td>gBifid-R</td>
<td>GGT GTT CTT CCC GAT ATC TAC A</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lac1</td>
<td>AGC AGT AGG GAA TCT TCC A</td>
<td>345</td>
<td>60°C</td>
</tr>
<tr>
<td>Lac2</td>
<td>ATT YCA CCG CTA CAC ATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (<em>Clostridium</em> cluster I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI-F1</td>
<td>TAC CHR AGG AGG AAG CCA C</td>
<td>232</td>
<td>63°C</td>
</tr>
<tr>
<td>CI-R2</td>
<td>GTT CTT CCT AAT CTC TAC GCA T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III (<em>Clostridium</em> cluster XIVab)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXIV-F1</td>
<td>GAW GAA GTA TYT CGG TAT GT</td>
<td>150</td>
<td>52°C</td>
</tr>
<tr>
<td>CXIV-R2</td>
<td>CTA CGC WCC CTT TAC AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probe-II (<em>C. bolteae</em>)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.bol-F</td>
<td>CAG GTG GTG CAT GGT TGT CGT CAG</td>
<td>157</td>
<td>58°C</td>
</tr>
<tr>
<td>C.bol-R</td>
<td>CCT CTT GAC CGG CGT GT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

13
4. Data and Instrumentation

**Design** – All neurotypical and autistic participants in this prospective, 2-visit, descriptive study were recruited according to IRB approved procedures mentioned above and asked to provide stool samples as described above under procedures.

**Instrumentation and Data Analysis** – The collected stool samples underwent DNA extraction and real-time PCR in the Department of Animal Sciences, Drs. Yu and Morrison’s lab. The extraction method used was developed by Drs. Yu and Morrison, and demonstrated to be an improved PCR-quality community DNA extraction has been published by the same OSU Animal Science group (12). The molecular characterization of the stool samples determined quantifiable amounts of beneficial bacteria, *Bifidobacterium* and *Lactobacillus*, as well as the pathogenic bacteria, *Clostridia* (*C. cluster I, C. cluster XIV, and C. bolteae*), in both autistic children and neurotypical children. Two-sample t-tests were used to determine whether differences existed in the five bacteria between the two groups of participants. As stated in the hypotheses above, it was anticipated that less *Bifidobacterium* and *Lactobacillus* would be present in the ASD samples as compared to the controls whereas an elevation of *Clostridia* species was expected. One-sided tests were used to remain consistent with these hypotheses and a Bonferroni adjustment was used to protect Type I error. The outcomes were log10-transformed to accommodate the assumptions of the tests, and analyzed by two-tailed *unpaired* t-tests (GraphPad Software, San Diego, CA). Significance was declared at $p \leq 0.05$. 

---

\[a\] Probe-I is an internal universal probe that corresponds to a region of the 16S rRNA gene that is conserved in all eubacteria (16); it was used with two sets of species (17, 18) and cluster-specific primers (3). Probe-II was used with *C. bolteae*-specific primer set (3). *Clostridium* clusters are as described in reference 19.

\[b\] Primer annealing temp
CHAPTER 3

RESULTS

Results are displayed as raw data expressed in relative abundance (Table 2) and means of real-time PCRs of each bacterial species characterized and analyzed (Figure 1 and 2).

TABLE 2. Bacteria detection in stool samples of autistic and neurotypical control children by real-time PCR

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Bifidobacteria</th>
<th>Lactobacilli</th>
<th>Clostridia cluster I</th>
<th>Clostridia cluster XIV</th>
<th>Clostridium bolteae</th>
</tr>
</thead>
<tbody>
<tr>
<td>T05</td>
<td>4.16</td>
<td>0.97</td>
<td>3.16</td>
<td>5.45</td>
<td>2.85</td>
</tr>
<tr>
<td>T06</td>
<td>4.09</td>
<td>2.57</td>
<td>3.39</td>
<td>5.70</td>
<td>2.06</td>
</tr>
<tr>
<td>T07</td>
<td>4.29</td>
<td>2.72</td>
<td>3.42</td>
<td>5.42</td>
<td>2.16</td>
</tr>
<tr>
<td>T08</td>
<td>4.37</td>
<td>2.06</td>
<td>3.29</td>
<td>5.67</td>
<td>2.35</td>
</tr>
<tr>
<td>T09</td>
<td>4.05</td>
<td>2.03</td>
<td>3.52</td>
<td>5.38</td>
<td>2.20</td>
</tr>
<tr>
<td>T10</td>
<td>4.56</td>
<td>1.23</td>
<td>3.26</td>
<td>5.50</td>
<td>2.53</td>
</tr>
<tr>
<td>T11</td>
<td>5.13</td>
<td>1.57</td>
<td>3.48</td>
<td>5.41</td>
<td>2.25</td>
</tr>
<tr>
<td>T12</td>
<td>4.88</td>
<td>1.84</td>
<td>3.74</td>
<td>5.38</td>
<td>2.70</td>
</tr>
<tr>
<td>T13</td>
<td>4.20</td>
<td>0.88</td>
<td>3.33</td>
<td>5.34</td>
<td>2.21</td>
</tr>
<tr>
<td>T14</td>
<td>5.35</td>
<td>1.77</td>
<td>2.79</td>
<td>5.62</td>
<td>2.89</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>4.5 ± 0.15</td>
<td>1.76 ± 0.12</td>
<td>3.34 ± 0.079</td>
<td>5.49 ± 0.04</td>
<td>2.42 ± 0.09</td>
</tr>
<tr>
<td>A01</td>
<td>4.61</td>
<td>1.35</td>
<td>3.49</td>
<td>5.53</td>
<td>2.72</td>
</tr>
<tr>
<td>A02</td>
<td>4.72</td>
<td>0.47</td>
<td>2.38</td>
<td>5.08</td>
<td>3.10</td>
</tr>
<tr>
<td>A06</td>
<td>4.50</td>
<td>1.03</td>
<td>2.31</td>
<td>5.60</td>
<td>3.46</td>
</tr>
<tr>
<td>A07</td>
<td>4.35</td>
<td>0.97</td>
<td>4.66</td>
<td>5.77</td>
<td>3.65</td>
</tr>
<tr>
<td>A14</td>
<td>4.38</td>
<td>1.39</td>
<td>2.98</td>
<td>5.51</td>
<td>3.20</td>
</tr>
<tr>
<td>A17</td>
<td>3.90</td>
<td>1.54</td>
<td>3.42</td>
<td>5.37</td>
<td>2.82</td>
</tr>
<tr>
<td>A22</td>
<td>4.56</td>
<td>0.94</td>
<td>2.52</td>
<td>4.92</td>
<td>1.32</td>
</tr>
<tr>
<td>A28</td>
<td>4.51</td>
<td>1.13</td>
<td>4.25</td>
<td>5.39</td>
<td>2.39</td>
</tr>
<tr>
<td>A32</td>
<td>4.33</td>
<td>0.03</td>
<td>3.30</td>
<td>5.32</td>
<td>3.74</td>
</tr>
<tr>
<td>A33</td>
<td>4.75</td>
<td>0.98</td>
<td>3.81</td>
<td>5.10</td>
<td>3.53</td>
</tr>
<tr>
<td>A37</td>
<td>3.67</td>
<td>1.37</td>
<td>3.48</td>
<td>5.47</td>
<td>3.15</td>
</tr>
<tr>
<td>A38</td>
<td>3.78</td>
<td>1.82</td>
<td>3.19</td>
<td>4.99</td>
<td>3.91</td>
</tr>
<tr>
<td>A39</td>
<td>3.31</td>
<td>0.91</td>
<td>3.84</td>
<td>5.64</td>
<td>4.35</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>4.26 ± 0.12</td>
<td>1.07 ± 0.13</td>
<td>3.36 ± 0.19</td>
<td>5.36 ± 0.07</td>
<td>3.18 ± 0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data expressed as relative abundance; copies per million copies (cpcm) of bacterial rrs genes; SEM, standard error of the mean.

<sup>b</sup>T, neurotypical children; A, autistic children.
Specific Aim 1: When compared to neurotypical controls, children with ASD revealed significantly lower amounts of *Lactobacilli* (p = 0.0059). No significant differences were found in the levels of *Bifidobactricia* (Figure 1).

FIGURE 1. Means of real-time PCR of *Bifidobacteria* and *Lactobacilli* detected in stool samples of children with ASD compared to neurotypical controls. P values of significance displayed.
Specific Aim 2: When compared to neurotypical controls, children with ASD revealed significantly higher amounts of *C. bolteae* \((p = 0.0079)\). No significant differences were found in the levels of *C. cluster I and XIV* (Figure 2).

FIGURE 2. Means of real-time PCR of *Clostridia species* (C. cluster I, C. cluster XIV, C. *bolteae*) detected in stool samples of children with ASD compared to neurotypical controls. P values of significance displayed.
DISCUSSION

The purpose of this study was to determine if the gut integrity of children with autism is compromised by a lower amount of beneficial microflora allowing for a higher amount of pathogenic microflora in the autistic gut. This study aimed to reproduce results similar to that of the Song et al (3) study’s findings. The study by Song et al (3) demonstrated the advantages of using real-time PCR in the molecular characterization of stool samples. To our knowledge, this research team is the only group that also incorporated Drs. Yu and Morrison’s improved DNA extraction technique (12) as well as real-time PCR in the molecular characterization process.

Analysis of the data collected from real-time PCR assays of children with autism compared to neurotypical controls suggest that children with autism may possess significantly lower counts of beneficial bacteria (Lactobacilli) and significantly higher counts of pathogenic bacteria (C. bolteae). No significant differences were found in the amounts of Bifidobacteria, C. cluster I, or C. cluster XIV.

Limitations of this study include our small sample size (n=23), large age range (3-9), and difficulties obtaining age and gender-matched controls. Although our eligibility criteria for neurotypical children included only children of normal health status and without gastrointestinal diagnoses or symptoms, there is no threshold or standard for the amount of beneficial or pathogenic bacteria known to inhabit the intrinsic microflora. Thus, we are forced to use the gut microflora of neurotypical children, whether optimal or not is unknown, when comparing to autistic children.

Several reports document the apparent parallel onset of autistic symptoms with gastrointestinal complications (4-6, 8), which has been associated with abnormalities in their gut mucosa (5, 6). The etiology of these abnormalities is not well understood in the literature today,
but it has been suggested that the imbalance of gastrointestinal microflora, or dysbiosis, in children with autism is related to autistic behaviors. Some researchers suggest that the *Clostridia* species, neurotoxin-producing bacteria, are responsible for or at least contribute to exacerbation of autistic behaviors (5, 6). Further research is necessary to determine the role that compromised gut integrity plays in autism spectrum disorders.

A study by Sandler et al (11) found marked improvement in communication, social skills, behavior, and gastrointestinal manifestations in children with autism following short-term treatment with antibiotics; however, all but one autistic participant regressed towards baseline following termination of the treatment. This suggests an even stronger link between the gut microflora and autistic symptoms (6). Antibiotics are not indicated for use long term in this population due to obvious side effects. This presents an indication for future research into the potential benefits of prebiotics and probiotics in this population. These dietary supplements (prebiotics) feed the beneficial microflora. When the beneficial bacteria ferment the fructooligosaccharides in prebiotics, it changes the pH of the intestinal track, making a very inhospitable environment for pathogenic bacteria (*Clostridia*) to thrive or survive. Research into the potential beneficial effects of these dietary supplements in children with autism may shed insight into a proactive, long-term option for this population.

Because the etiology of autism spectrum disorders remains unclear, we are not prepared with effective means of treatment or prevention. The results of this research study support the theory that children with autism posses an imbalance of beneficial and pathogenic microflora in their gut mucosa. Future studies with larger sample sizes as well as age and gender matched controls are necessary to determine if compromised gut integrity exists among children with autism. It would be worthwhile to have a retrospective questionnaire to determine if the autistic
child’s disorder arose in conjunction with gastrointestinal distress and/or complications. Understanding the role of dysbiosis in this population could lend to prospective approaches to this idiopathic disorder. As the prevalence of autism continues to rise, research efforts must focus on the most promising solutions to accommodate these disorders.
REFERENCES


